

BACTERIAL REVERSE MUTATION TEST



FINAL REPORT

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STUDY DIRECTOR COMPLIANCE STATEMENT

The study met with the technical requirements of the protocol, and all applicable guidance and regulations, which included the Good Laboratory Practice for Non-clinical Laboratory Studies (FDA, 21 CFR, Part 58), Good Laboratory Practice for Non-clinical Laboratory Studies (DOH, R.O.C., 3rd ed., 2006), and OECD Principles on Good Laboratory Practice (TAF OECD GLP Compliance, No. 1, 1997). During the testing, there was one amendment from the approved study protocol (amendment sheet No. PSA11070003-01) and no adverse problems that would affect the integrity of the results or the interpretation of our conclusion.

STUDY DIRECTOR:

Jian-Yu Lin, M.S.

DATE

QUALITY ASSURANCE STATEMENT

This report has been reviewed by the Quality Assurance Unit and accurately reflects the raw data. The following study specific inspections were conducted and findings reported to study director and management.

INSPECTIONS	Date of Inspection	Date Reported	Date Reported
		-Study Director	-Facility Manager

QUALITY ASSURANCE:

Wei-shiun Huang, QAU Manager

DATE

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BACTERIAL REVERSE MUTATION TEST

ABSTRACT

“Bacterial Reverse Mutation Test” was used in this study to evaluate the genotoxicity of [REDACTED] in accordance with OECD Guideline for the testing of chemicals #471 (1997): Bacterial reverse mutation test, and the operation was executed according to the standard operation procedure of Level Biotechnology Inc. (SOP: MP007-04).

Salmonella typhimurium TA100 was chosen and 5 mg/plate of [REDACTED] was used as highest dose in “Dose Range Finding Test” to determine the testing dose for “Bacterial Reverse Mutation Test”. The result of “Dose Range Finding Test” indicated that [REDACTED] showed non-cytotoxic and non-mutagenic effects in *Salmonella typhimurium* TA100.

According to the result of “Dose Range Finding Test”, 5 mg/plate was chosen as the highest dose and the other four doses (2.5, 1.25, 0.625, 0.313 mg/plate) were then determined for five *Salmonella typhimurium* strains “Bacterial Reverse Mutation Test”. *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535, and TA1537 were used, and plate incorporation method in the presence and absence of S9 metabolic mixture were applied in this test.

Based on the results of this test, no significant increase in the numbers of revertant colonies was observed. The test article [REDACTED] did not present genotoxic effect at all concentrations of testing strains in the condition of both presence and absence of S9 metabolic mixture. In conclusion, [REDACTED] was non-genotoxic in the testing system applied in this study.

STATEMENT: THE TESTING RESULT IS EFFECTIVE FOR SUBMITTED SAMPLE ONLY, AND SHALL NOT BE EXCERPTED FROM THE CONTENTS OF THIS REPORT WITHOUT THE WRITTEN APPROVAL OF THE TESTING FACILITY.

1. PURPOSE

The purpose of this assay was to evaluate the genotoxic potential of [REDACTED]
The testing methodology and evaluation were in accordance with OECD Guideline for the testing of chemicals #471 (1997): Bacterial reverse mutation test.

2. GENERAL INFORMATION

2.1 Study Number: PSA11070003

2.2 Sponsor:

Sponsor Name: [REDACTED]

Address: [REDACTED]

Representative: [REDACTED]

2.3 Testing Facility

Level Biotechnology Inc. Preclinical Testing Center
No. 80, Lane 169, Kangning St., Hsi-Chih Dist.,
New Taipei City 221, Taiwan (R.O.C.)

2.4 Testing Laboratory

Level Biotechnology Inc. Preclinical Testing Center
Testing Room number: B106
Data Analysis: Room B302

2.5 Personnel

Study director: Jian-Yu Lin, M.S.

Study associate: Hui-Wen Zhuang, M.S., Yu-Chung Chiao, Ph.D.,
Yu-Wen Huang, M.S.

2.6 Data Retention

The raw data, documents, records, protocol and the final report generated from this study were archived in the archive room of Level Biotechnology Inc., Preclinical Testing Center for 5 years. The test article was sampled and preserved in Quality Assurance Unit for 2 years. Extension of the conservation, destroying or receding of these records and raw data after the retaining duration would be performed according to the request from the sponsor.

3. STUDY SCHEDULE

3.1 Experimental starting date: Jul. 20, 2011

3.2 Bacterial strains preparation and identification: Jul. 20, 2011 ~ Jul. 29, 2011

3.3 Dose range finding test (in TA100): Aug. 01, 2011 ~ Aug. 05, 2011

3.4 Bacterial reverse mutation test (5 strains): Aug. 08, 2011 ~ Aug. 12, 2011

3.5 Experimental completion date: Aug. 12, 2011

4. TEST SYSTEM

4.1 Bacterial Strains :

Salmonella typhimurium strain TA98, TA100, TA102, TA1535, and TA1537

Genotype of bacterial strains

Strains	Histidine mutation	LPS	Repair ($\Delta uvrB^1$)	R-factor	pAQ1 plasmid
TA98	<i>hisD3052</i>	<i>rfa</i>	$\Delta uvrB$	+R	—
TA100	<i>hisG46</i>	<i>rfa</i>	$\Delta uvrB$	+R	—
TA102	<i>hisG428</i>	<i>rfa</i>	+ ²	+R	+ ³
TA1535	<i>hisG46</i>	<i>rfa</i>	$\Delta uvrB$	—R	—
TA1537	<i>hisC3076</i>	<i>rfa</i>	$\Delta uvrB$	—R	—

1 : " Δ " means gene deletion

2 : "+" means wild-type gene

3 : Insertion of the mutation *hisG428* on the multicopy plasmid pAQ1

4.2 Bacterial Source:

Moltox Inc., U.S.A.

4.3 Genotype Confirmation:

The genotype confirmation of bacterial strains was performed according to SOP: MP007-04 before testing.

5. TEST AND CONTROL ARTICLES

5.1 Test Article

Name: [REDACTED]

Source: [REDACTED]

Sample delivery date: Jul. 07, 2011

Sample inspection complete date: Jul. 08, 2011

Lot/Batch No.: s25211001

CAS No.: [REDACTED]

Major ingredients: [REDACTED]

Purity of active ingredient: 99%~100%

Certification of analysis attached: Yes

Physical description: White crystalline powder, non-sterile, and odorless

Solvent and solubility: (g in 100ml solvent @ 25°C)

Toluene: 25

Acetone: 35

Ethyl Acetate: 25

Dichloromethane: >50

Water: <0.2

pH value: n/a

Storage condition: room temperature, keep container tightly closed when not in use and during transport

Expiration date: Jun. 26, 2013

Intended use: n/a

Test Article No.: T110703

5.2 Control Article

A. Positive control

The positive control reference substances were selected on the basis of the type of bacterial strains used. The following chemicals were used as positive control substances for assays without or with metabolic activation.

Strain	S9	Positive control, µg/plate
TA98	—	2-nitrofluorene (2-NF), 1 µg/plate
TA100		Sodium azide (SA), 1 µg/plate
TA102		Mitomycin C (MMC), 0.2 µg/plate
TA1535		SA, 1 µg/plate
TA1537		9-aminoacridine (9-AA), 50 µg/plate
TA98	+	2-aminoanthracene (2-AA), 1 µg/plate
TA100		Benzo[a]pyrene (BP), 1 µg/plate
TA102		2-AA, 5 µg/plate
TA1535		2-AA, 5 µg/plate
TA1537		2-AA, 5 µg/plate

Water for injection was used as solvent of 9-AA, SA and MMC.

DMSO was used as solvent of 2-NF, 2-AA and BP.

B. Negative control

Name: Sterile water

Source: Biological Industries, 03-055-1A

Lot/Batch No.: 1007382

Storage condition: Room temperature

Expiration date: Feb, 2013

C. Vehicle control

Name: Acetone

Source: J.T. Baker 9006-01

Lot/Batch No.: J09B07

Expiration date: Dec. 21, 2015

Storage condition: Room temperature

6. EXPERIMENTAL DESIGN AND METHODOLOGY

6.1 Justification for choice of vehicle

The vehicle should not be suspected of chemical reaction with the test article and should be compatible with the survival of the bacteria and the S9 activity.

6.2 Test Article Preparation (SOP: MP023-01)

The test article [REDACTED] was suspended to the stock concentration of 100 mg/mL by acetone. 5 mg/plate of [REDACTED] was used as the highest dose in "Dose range finding test". Insolubility should be assessed as precipitation in the final mixture under the actual test conditions and evident to the unaided eyes, and the precipitation should not interfere the scoring. In the groups with metabolic activation, 0.5 mL of metabolic mixture was used instead of 0.2M Phosphate buffer stated in 6.4.B.

6.3 Metabolic Activation System (S9)

The test article was exposed to the S9 mixture consisted of S9 fraction (Aroclor 1254-induced; Moltox, BOONE, U.S.A.) and cofactor, which was used to mimic the metabolic activation system. The S9 mixture was added at final concentration of 0.94% (v/v).

6.4 Dose Range Finding Test in TA100

A. Dose range finding test of test article in TA100 was performed according to SOP: MP007-04. The testing concentrations was designed as below:

Strain	S9	Groups	Dose
TA100	—	Negative control (sterile water)	—
		Vehicle control (acetone)	—
		[REDACTED]	5 mg/plate
			2.5 mg/plate
			1.25 mg/plate
			0.625 mg/plate
			0.313 mg/plate
		Positive control (Sodium azide)	1 µg/plate

Sodium azide (Sigma S2002, Lot: 098K0052, Exp. Date: Aug. 23, 2011)

B. Plate incorporation method was employed and performed as following:

(a) The components were added sequentially according to SOP: MP007-04:

- (1) 0.5 mL of 0.2 M Phosphate buffer, pH=7.4 (without S9 metabolic activation).
 - (2) 0.05 mL of each testing concentrations of [REDACTED], negative, vehicle, or positive control solution.
 - (3) 0.1 mL of overnight culture of the *Salmonella typhimurium* strains (containing approximately $1\sim2 \times 10^9$ cells/mL).
 - (4) 2 mL of molten top agar (with 0.5mM histidine/biotin).
- (b) The contents of each tube were mixed and poured onto the surface of minimal glucose agar plates.
 - (c) When the top agar has been solidified, the plates were inverted and placed in a $37 \pm 1^\circ\text{C}$ incubator for 48 ~ 72 hours. The colonies were then counted.
 - (d) All groups, including positive, negative, vehicle, and the five dosage testing groups, were performed in triplicates.
- C. The preliminary dose range finding test was carried out to decide the appropriate starting dosage. The other four doses were used with half intervals between test points as described in 6.4.

6.5 Testing Procedures of Bacterial Reverse Mutation Test (5 strains)

- A. The methodology would be the same and the doses used below would be determined according to the result of dose range finding test.

Without S9 mix

Strains	S9	Groups	Dose (mg/plate)
TA98	—	Negative control (water)	—
		Vehicle control (acetone)	—
		[REDACTED]	5, 2.5, 1.25, 0.625, 0.313
		Positive control (2-nitrofluorene)	1 µg/plate
TA100	—	Negative control (water)	—
		Vehicle control (acetone)	—
		[REDACTED]	5, 2.5, 1.25, 0.625, 0.313
		Positive control (Sodium azide)	1 µg/plate
TA102	—	Negative control (water)	—
		Vehicle control (acetone)	—
		[REDACTED]	5, 2.5, 1.25, 0.625, 0.313
		Positive control (Mitomycin C)	0.2 µg/plate
TA1535	—	Negative control (water)	—

		Vehicle control (acetone)	—
		██████████	5, 2.5, 1.25, 0.625, 0.313
		Positive control (Sodium azide)	1 µg/plate
TA1537		Negative control (water)	—
		Vehicle control (acetone)	—
		██████████	5, 2.5, 1.25, 0.625, 0.313
		Positive control (9-aminoacridine)	50 µg/plate
	2-nitrofluorene (Aldrich N16754, Lot: 03926DC, Exp. Date: Aug. 23, 2011)		
Sodium azide (Sigma S2002, Lot: 098K0052, Exp. Date: Aug. 23, 2011)			
Mitomycin C (Sigma M4287, Lot: 010M0665, Exp. Date: Aug. 23, 2011)			
9-aminoacridine (Aldrich A38401, Lot: S32398-468, Exp. Date: Aug. 23, 2011)			
With S9 mix			
Strains	S9	Groups	Dose (mg/plate)
TA98		Negative control (water)	—
		Vehicle control (acetone)	—
		██████████	5, 2.5, 1.25, 0.625, 0.313
		Positive control (2-aminoanthracene)	1 µg/plate
TA100		Negative control (water)	—
		Vehicle control (acetone)	—
		██████████	5, 2.5, 1.25, 0.625, 0.313
		Positive control (Benzo[a]pyrene)	1 µg/plate
TA102	+	Negative control (water)	—
		Vehicle control (acetone)	—
		██████████	5, 2.5, 1.25, 0.625, 0.313
		Positive control (2-aminoanthracene)	0.2 µg/plate
TA1535		Negative control (water)	—
		Vehicle control (acetone)	—
		██████████	5, 2.5, 1.25, 0.625, 0.313
		Positive control (2-aminoanthracene)	1 µg/plate
TA1537		Negative control (water)	—
		Vehicle control (acetone)	—
		██████████	5, 2.5, 1.25, 0.625, 0.313
		Positive control (2-aminoanthracene)	50 µg/plate

2-aminoanthracene (Aldrich A38800, Lot: S17744-374, Exp. Date: Aug. 23, 2011)

Benzo[a]pyrene (Sigma B1760, Lot: 097K1293, Exp. Date: Aug. 23, 2011)

B. Plate incorporation method was employed and performed as following:

- (a) The components were added sequentially according to SOP: MP007-04:
 - (1) 0.5 mL of 0.2 M Phosphate buffer, pH=7.4 (without S9 metabolic activation).
 - (2) 0.05 mL of each testing concentrations of [REDACTED], negative, vehicle, or positive control solution.
 - (3) 0.1 mL of overnight culture of the *Salmonella typhimurium* strains (containing approximately $1\sim2 \times 10^9$ cells/mL).
 - (4) 2 mL of molten top agar (with 0.5mM histidine/biotin).
- (b) The contents of each tube were mixed and poured onto the surface of minimal glucose agar plates.
- (c) When the top agar has been solidified, the plates were inverted and placed in a $37 \pm 1^\circ\text{C}$ incubator for 48 ~ 72 hours. The colonies were then counted.
- (d) All groups, including positive, negative, vehicle, and the five dosage testing groups, were performed in triplicates.

In the groups with metabolic activation, 0.5 mL of S9 metabolic mixture was used instead of 0.5 mL of 0.2M Phosphate buffer stated in 6.5.B(a)(1).

7. EVALUATION OF TEST RESULTS

7.1 The raw data of revertant colony values were represented with Mean \pm S.D.

7.2 Cell toxicity determination

A. A cytotoxic effect was concluded when a decrease in revertant colonies over the negative/vehicle control was lower than 0.5-fold, loss of bacterial lawn, or pin colony appeared.

B. Plates would be labeled and excluded from statistics while cytotoxic effect occurred.

7.3 An increase in revertants over the negative control would be as the cut-off between a mutagenic and non-mutagenic response:

A. TA98, TA100 and TA102: more than two-fold increase in revertants over the negative/vehicle control, then the test articles would be considered as a potential mutagen.

B. TA1535 and TA1537: more than three-fold increase in revertants over the negative/vehicle control, then the test articles would be considered as a potential mutagen.

- 7.4** If the test article was considered as a potential mutagen, the raw data would be further analyzed by ANOVA to evaluate the difference between negative/vehicle control group and test article groups, and $p < 0.05$ indicates the significant difference.
- 7.5** If the data showed statistically significant, then to evaluate the dose-related response in the numbers of revertant colonies on the test article groups as compared with the vehicle control group. Once dose-related response was confirmed, the test articles would be considered as a mutagen.

Strain	Revertants number	Dose-dependent response	Result
TA98	two-fold increase over the negative/vehicle control	yes	mutagenic
TA100 TA102		no	non-mutagenic
TA1535	three-fold increase over the negative/vehicle control	yes	mutagenic
TA1537		no	non-mutagenic

8. RESULT AND DISCUSSION

8.1 The genotyping of *Salmonella typhimurium* strains

The result was organized in table 1 and Appendix D-1

The genotypes of five *Salmonella typhimurium* strains (TA98, TA100, TA102, TA1535, and TA1537) for this study were identified. The historic control data of bacterial reverse mutation test was listed in Appendix C.

8.2 Result of Dose Range Finding Test in TA100

Five doses (5, 2.5, 1.25, 0.625, and 0.313 mg/plate) of [REDACTED] were used, and no cytotoxic and mutagenic effects occurred. Based on the result, 5 mg/plate of [REDACTED] was determined as the highest dose for five strains bacterial reverse mutation test. All the raw data were authentic and organized in Table 2 and Appendix D-2.

8.3 Result of Bacterial Reverse Mutation Test (5 strains)

- A.** Based on the result of dose range finding test, the concentrations of 5 mg/plate of [REDACTED] was used as the highest dose and remaining four doses 2.5, 1.25, 0.625, and 0.313 mg/plate were used for bacterial reverse mutation test in five *Salmonella typhimurium* strains.
- B.** This testing system was validated and authentic. The testing result organized in Table 3, and Appendix D-3.
- (a) The numbers of revertant colony in negative control groups of each strain

were in the range of historic control data.

- (b) The revertant colonies in positive control group were more than two times (in TA98, TA100, and TA102) and three times (in TA1535 and TA1537) over negative control groups.

C. No significant increase in the numbers of revertant colonies was observed at all concentrations of testing strains in both presence and absence of S9 metabolic mixture, indicated the test article [REDACTED] did not present genotoxic effect.

9. CONCLUSION

This test was performed in accordance with OECD Guideline for the testing of chemicals #471 (1997): Bacterial reverse mutation test, to evaluate the genotoxic potential of [REDACTED]

According to the numbers of revertant colonies at all the testing conditions of the five strains used in this study (i.e. TA98, TA100, TA102, TA1535, and TA1537), [REDACTED] did not present genotoxic effect.

10. REFERENCES

- 10.1 Ames, B. N. and Maron, D. M. (1983) Revised methods for the Salmonella mutagenicity test. Mutation Res. 113: 173-215.
- 10.2 FDA Redbook (2000) Toxicological principles for the safety assessment of food ingredients.
- 10.3 Health Effects Test Guidelines, OPPTS 870.5100 (1998) Bacterial Reverse Mutation Test. United States Environmental Protection Agency.
- 10.4 Maron, D., Katzenellenbogen, J., and Ames, B. N. (1981). Compatibility of Organic Solvents with the Salmonella/Microsome Test. Mutation Res. 88 343-350.
- 10.5 Mortelmans, K. and Zeiger, E. (2000) The Ames Salmonella/microsome mutagenicity assay. Mutation Res. 455: 29-60.
- 10.6 OECD Guideline for the testing of chemicals #471: Bacterial reverse mutation test, (1997).


Table 1. Result of Genotyping of *Salmonella typhimurium* strains

<i>Salmonella typhimurium</i> strains	Histidine requirement		UV sensitivity	Crystal violet sensitivity	Ampicillin resistance	Tetracycline resistance	Spontaneous revertants
	His+ Bio+ Plate	His- Bio+ Plate	UV irradiated	Zone of growth inhibition*	Ampicillin plate	Ampicillin Tetracycline plate	without S9
TA98	+	—	—	+	+	—	24.0 ± 1.7
TA100	+	—	—	+	+	—	153.7 ± 12.4
TA102	+	—	+	+	+	+	352.0 ± 12.5
TA1535	+	—	—	+	—	—	12.3 ± 1.5
TA1537	+	—	—	+	—	—	14.3 ± 1.2

Remark:

* + : a clear zone of inhibition appeared around the disc



Table 2. Result of Dose Range Finding Test (in TA100)

Dose (mg / plate)		Number of revertants /plate (Mean \pm S.D., n=3)		
		without S9 mixture		
Negative control (sterile water)		161.7	\pm	5.9
Positive control (Sodium azide, 1 μ g/plate)		412.0	\pm	26.5*
Vehicle control (Acetone)		173.7	\pm	15.3
	5	156.7	\pm	7.2
	2.5	158.3	\pm	19.2
	1.25	181.3	\pm	7.2
	0.625	169.7	\pm	11.6
	0.313	162.7	\pm	20.2

Remark:

1.*: more than two-fold increase in revertants over the negative control

Table 3. Result of Bacterial Reverse Mutation Test (5 strains)

Group (mg/plate)		Number of revertants /plate (without S9 activator, Mean \pm S.D., n=3)				
		TA98	TA100	TA102	TA1535	TA1537
Negative control		43.7 \pm 7.5	167.3 \pm 9.6	236.7 \pm 9.1	8.0 \pm 0.0 ^a	13.0 \pm 2.6
Positive control		156.3 \pm 24.0*	633.3 \pm 19.7*	1872.0 \pm 169.3*	478.7 \pm 39.7*	181.3 \pm 53.8*
Vehicle control		24.3 \pm 5.1	169.0 \pm 7.2	265.0 \pm 20.5	15.3 \pm 0.6	15.7 \pm 1.5
	5	26.7 \pm 6.4	170.7 \pm 12.2	264.0 \pm 7.0	12.0 \pm 2.6	21.0 \pm 4.4
	2.5	24.3 \pm 6.8	167.0 \pm 6.2	296.0 \pm 7.0	9.7 \pm 2.1	19.0 \pm 4.0
	1.25	25.3 \pm 3.5	159.3 \pm 12.7	304.7 \pm 12.1	13.0 \pm 5.6	18.3 \pm 3.1
	0.625	23.0 \pm 1.0	169.0 \pm 16.1	299.3 \pm 3.5	10.0 \pm 2.0	18.7 \pm 2.9
	0.313	23.7 \pm 2.1	167.3 \pm 13.0	289.0 \pm 18.0	6.0 \pm 1.4 ^a	22.3 \pm 2.5
Group (mg/plate)		Number of revertants /plate (with S9 activator, Mean \pm S.D., n=3)				
Negative control		48.7 \pm 2.9	148.7 \pm 16.3	381.3 \pm 16.5	10.3 \pm 4.5	9.3 \pm 2.1
Positive control		781.3 \pm 72.1*	566.7 \pm 59.5*	988.0 \pm 51.1*	179.7 \pm 64.8*	358.0 \pm 45.2*
Vehicle control		37.0 \pm 1.0	142.0 \pm 6.2	386.7 \pm 41.0	9.7 \pm 2.5	11.3 \pm 4.5
	5	37.0 \pm 3.5	142.7 \pm 14.5	311.3 \pm 17.5	9.7 \pm 3.2	12.3 \pm 3.1
	2.5	36.3 \pm 4.0	133.7 \pm 6.4	377.3 \pm 17.0	10.7 \pm 1.5	13.7 \pm 0.6
	1.25	41.3 \pm 4.9	143.7 \pm 14.0	346.0 \pm 40.1	8.0 \pm 1.7	12.0 \pm 2.6
	0.625	39.7 \pm 5.5	137.3 \pm 10.3	374.7 \pm 13.2	9.0 \pm 1.0	10.7 \pm 1.5
	0.313	41.3 \pm 8.1	148.3 \pm 1.5	366.0 \pm 7.2	9.3 \pm 2.5	10.7 \pm 0.6

Remark:

1. The use of positive control substance for each strains was listed in 5.2A
2. *: more than two or three-fold increase in revertants over the negative control
3. a: n=2